APPENDIX F

DETERMINATION OF THE STEROL CONTENT IN MARGARINES, HALVARINES, DRESSINGS, FAT BLENDS AND STEROL FATTY ACID ESTER CONCENTRATES BY CAPILLAIRY GAS CHROMATOGRAPHY

Unilever

1. WARNING AND SAFETY PRECAUTIONS

The usual safety precautions for working with chemicals in laboratories apply.

2. SCOPE

The method describes a gas chromatographic procedure for the determination of the total sterol content in margarines, halvarines, dressings, fats or fat blends and sterol ester concentrates. The method is designed for total sterol levels of approx. 10 % in margarines, fats and fat blends, 8 % in halvarines, 3% - 10% in dressings, and approx. 60 % in sterol ester concentrates. The sterols should originate from soybean oil or similar vegetable oils and should be present only as sterol fatty acid esters or free sterols.
The method is not suitable for the identification of unknown sterols.

3. NORMATIVE REFERENCES

The gasses that are required for the gas chromatographic process are purified by means of standard filters to remove any impurities.

ISO 3696, 1987, water for analytical laboratory use. Specifications and test methods.

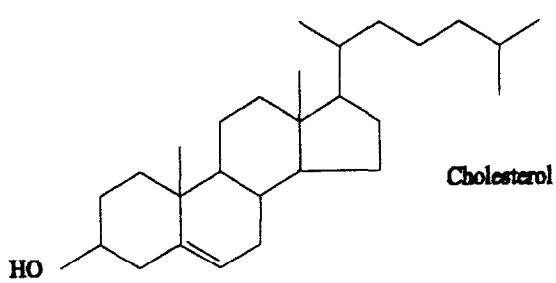
4. PRINCIPLE

For quantification an internal standard is added to the sample. The sample is saponified and the unsaponifiable part is extracted. The extract is analyzed by means of gas chromatography using a non-polar stationary phase capillary column.

The recommended injection technique is 'cold on column'.
Detection takes place by means of a flame ionization detector.

6. REACTIONS AND ANALYTES

6.1 General



Cholesterol

An example of a sterol is shown above.

6.2 **Saponification of triglycerides**

Triglyceride + KOH -----> Glycerol + Soap

6.3 **Saponification of sterol esters**

Sterolester + KOH -----> Sterol + Soap

7. REAGENTS AND STANDARDS

Unless otherwise indicated, use chemicals with an Analytical grade of purification.

7.1 Chemicals

7.1.1 Ethanol, CAS no. [64-17-5] — $c[C_2H_5OH] > 99.8\ \%$
Flammable, avoid flames and sparks.

7.1.2 Potassium hydroxide, solid, CAS no. [1310-58-3] — $c[KOH] > 85\ \%$
Corrosive, avoid swallowing and contact with skin.

7.1.3 tert-Butyl methyl ether (MTBE), CAS no. [1634-04-4] — $c[C_5H_{12}O] > 99.8\ \%$
Harmful, avoid inhalation, swallowing and contact with skin.
Flammable, avoid flames and sparks.

7.1.4 n-Heptane, HPLC grade, CAS no. [142-82-5] — $c[C_7H_{16}] > 99\ \%$
Harmful, avoid inhalation, swallowing and contact with skin.
Flammable, avoid flames and sparks.

7.1.5 Sodium sulphate anhydrous, CAS no. [7757-82-6] — $c[Na_2SO_4] > 99\ \%$
Avoid swallowing, irritating to eyes.

7.1.6 Demi-water (class 3)

7.2 Standards

7.2.1 ß-Cholestanol, CAS no. [80-97-7] — $c[C_{27}H_{48}O] > 95\ \%$
(5□-Cholestane-3ß-ol)
E.g. supplier Sigma, Fluka, Aldrich.

Purity check
Before ß-Cholestanol is used the purity has to be confirmed. This purity check has to be carried out with every new batch.

Weigh approx. 25 mg ß-Cholestanol into a 50 ml measuring flask. Dissolve in and fill up to volume with MTBE (7.1.3) and homogenise the solution. Transfer 20 µl from this solution to a GC autosampler vial and add 1980 µl heptane (dilution 100 x). After dilution the GC vial is capped and shaken to obtain a homogeneous solution. This solution has to be analysed with the GC conditions as described in paragraph 8.
For quantification all peaks in the chromatogram have to be integrated. The purity is calculated as follows:

Purity ß-Cholestanol (%) = peak area ß-Cholestanol (%)

Carry out a correction for the internal standard purity in the calculation of sample concentrations.
Do not rely on the purity as provided by the supplier in the calculations for total sterol amount.

7.3 **Standard solutions**

7.3.1 ß-Cholestanol internal standard solution (solution A) □ 30 mg/ml. c[$C_{27}H_{48}O$] □ 30 mg/ml
Weigh approx. 300 mg ß-Cholestanol (7.2.1) accurately to 0.1 mg into a 10 ml measuring flask. Dissolve in and fill up to volume with MTBE (7.1.3). Divide the solution in small portions (about 1 ml, for example in GC vials) and store the internal standard solution in the freezer. The maximum shelf life of the solution is 6 months.

7.3.2 ß-Cholestanol internal standard solution (solution B) □ 100 mg/ml. c[$C_{27}H_{48}O$] □ 100 mg/ml
Weigh approx. 1000 mg ß-Cholestanol (7.2.1) accurately to 0.1 mg into a 10 ml measuring flask. Dissolve in and fill up to volume with MTBE (7.1.3). Divide the solution in small portions (about 1 ml, for example in GC vials) and store the internal standard solution in the freezer. The maximum shelf life of the solution is 6 months.

7.4 **Reagents**

7.4.1 Ethanolic Potassium Hydroxide Solution c[KOH] = 2 mol/l
Dissolve approx. 14 grams KOH (7.1.2) under nitrogen in 10 ml water and allow to cool to room temperature. After cooling fill up with ethanol (7.1.1) to 100 ml.
Keep this ethanolic KOH-solution under nitrogen in the refrigerator. The maximum shelf life of the solution is 3 months.
Flammable, avoid flames and sparks.
Corrosive, avoid inhalation, swallowing and contact with skin.

7.5 **Reagents for the gas chromatographic analysis**

7.5.1 Carrier gas
Hydrogen, purified over moisture filter, oxygen filter and organic matter (carbon) filter.

7.5.2 Flame gasses
Hydrogen, purified over moisture filter, oxygen filter and organic matter (carbon) filter.
Air, purified over moisture filter and organic matter (carbon) filter.

8. APPARATUS AND EQUIPMENT

Use standard laboratory equipment and requirements.
Also use:

8.1 Reaction-vials, 10 ml with silicon/Teflon discs (e.g. Pierce Reacti-vial^{tm}, 10 ml).

8.2 Glass jars, 20 ml with screw cap (e.g. Packard Econo Glass Vial^{tm}, 20 ml).

8.3 GC vials with sealable caps, suitable for autosampler.

8.4 Syringes, 100 and 500 µl (e.g. Camag Linomat syringes).

8.5 Gas chromatograph, suitable for capillary columns equipped with a:
- Flame ionisation detector (FID),
- Cold on column injector,
- Autosampler,
- Capillary column (8.6).

8.6 Capillary column:
- Precolumn: uncoated fused silica capillary, apolar deactivated, 1.0 m x 0.53 mm id (e.g. Interscience, HRGC precolumn, code 26060370).
- Analytical column: fused silica capillary coated with 100 % dimethylpolysiloxane (e.g. CP-Sil5-CB, DB-1, HP-1, OV-1, RTX-1 etc.).
  - Length 10 m,
  - Internal diameter 0.32 mm,
  - Film thickness 0.12 µm.

The precolumn is connected to the analytical column by means of a glass press fit connector.

8.7 GC Conditions:

Carrier gas flow:	2.2 ml/min (45.6 cm/sec)
Oven temperature programme:	60 °C (1 min), with 20 °C/min to 300 °C (3 min)
Detector Temperature:	320 °C
Injection volume:	0.5 µl
Electrometer range:	10°
Attenuation:	2^{4}

8.8 A chromatography data processing system.
(e.g. Perkin Elmer Nelson 2700 Turbochrom software on a PC (486 DX, 33MHz)).

9. SAMPLING AND PREPARATION

9.1 Sample preparation

9.1.1 *Sterol ester concentrates, fats and fat blends*
These samples should first be melted in an oven or microwave to obtain a homogenous clear liquid. Weigh approx. 50 mg sample accurately to 0.1 mg into the reaction-vial.

9.1.2 Margarine
The margarine samples require no special pretreatment.
Weigh approx. 50 mg of the sample accurately to 0.1 mg into the reaction-vial. Take a representative portion from the middle of the sample.

9.1.3 Halvarine
The halvarine samples require no special pretreatment.
Weigh approx. 50 mg sample accurately to 0.1 mg into the reaction-vial. Take a representative portion from the middle of the sample.

9.1.4 Dressings
As large a sample as possible, up to 8 ounces, is blended through a Silverson L4R for two minutes. If at least 2 ounces of sample is not available, the sample can be blended in a Waring Commercial Blender, using a cup size appropriate to the amount of sample available.

10. PROCEDURE

10.1 Safety precautions

All activities must be carried out in the fume cupboard.

10.2 Addition of internal standard

Add with a syringe (8.4) ß-Cholestanol solution to the sample into the reaction-vial as given in table 1.

Table 1: The amount of internal standard solution which must be added to the different sample types.

Sample	ß-Cholestanol solution	Volume (µl)
Sterol ester concentrate	B (c=100 mg/ml)	200
Fat or fatblend	A (c=30 mg/ml)	100
Margarine	A (c=30 mg/ml)	100
Dressings	A(c=30mg/ml)	100
Halvarine	A (c=30 mg/ml)	100

10.3 Saponification of the samples

10.3.1 Add 1 ml ethanolic potassium hydroxide solution (7.4.1).

10.3.2 Seal the reaction-vial with disc and cap and shake intensively for approx. 10 seconds by means of a vortex.

10.3.3 Reaction time
Margarines, halvarines, dressings, fats and fat blends and sterol ester concentrates.
Place the vial in a heating block at approx. 70 °C and saponify the sample during 50 minutes. The sample has to be shaken intensively by means of a vortex every 5 minutes. The last 5 minutes the vial is no longer shaken, in order to prevent sample loss upon opening the vial.

10.4 Extraction of sterols

10.4.1 Cool the sample to room temperature and open the vial.

10.4.2 Add 1 ml demi-water (7.1.6) and 5 ml heptane (7.1.4).

10.4.3 Close the reaction-vial with screw cap and disc and shake the vial intensively by means of a vortex for 0.5 minutes.

10.4.4 After separations of the phases transfer the upper (heptane-) phase by means of a Pasteur pipette into a glass jar (8.2).

10.4.5 Repeat the extraction of the reaction mixture 3 times with 3 ml heptane each. The heptane phases are also transferred into the glass jar.

10.4.6 Add 0.5-1.0 g sodium sulphate anhydrous (7.1.5) and shake the combined heptane extract in order to obtain a homogeneous dry solution.

10.5 GC analysis of the samples

10.5.1 The extracts must be diluted with heptane (7.1.4) for GC analysis as given in table 2. This dilution is carried out directly in the GC autosampler vial. The volumes given in table 2 are transferred to the GC autosampler vial by means of a Finn-pipette.

Table 2: Dilution before GC analysis.

Sample	Volume extract (□l)	Volume heptane (□l)	Dilution
Sterol ester concentrate	50	1950	40 x
Fat or fatblend	500	1500	4 x
Margarine	500	1500	4 x
Dressings	500	1500	4 x
Halvarine	500	1500	4 x

10.5.2 After dilution the filled GC vials are capped and shaken to obtain a homogeneous solution.

10.6 GC analyses

After creating the sequence file by means of the chromatography data processing system (8.8) the samples are placed in the autosampler and the GC analysis is started.

10.7 Identification of sterol peaks

The sterol peaks can be identified using the gas chromatogram given in appendix 3. Peaks with relative retention times from 0.98 till 1.13 (relative to □-Cholestanol as internal standard) are regarded as sterols and are included in the quantification. Please note that several sterols elute within the same relative retention time window. The method can not be used for unambiguous identification of individual sterols.

11. CALCULATION AND EXPRESSION OF RESULT

11.1 Individual sterols

Calculate the content of individual sterols -expressed in weight percent- in the sample as follows:

$$C[\text{Total sterol}] = \frac{C_{IS} * V_{IS} * A_{Sterol}}{A_{IS} * W_s * 10} * \frac{PURITY_{is}}{100}$$

in which:
C_{IS} = Concentration of internal standard (mg/ml).
V_{IS} = Volume of internal standard (□l).
A_{Sterol} = Peak area of the sterol.
A_{IS} = Peak area of the internal standard.
W_s = Weight of sample (mg).

For establishing the purity of the internal standard see paragraph 7.2.

11.2 Total sterols

Calculate the content of total sterols - expressed in weight percent - in the sample as follows:

$$C[\text{Total sterol}] = \frac{C_{IS} * V_{IS} * \Sigma A_{Sterol}}{A_{IS} * W_s * 10} * \frac{PURITY_{is}}{100}$$

in which:
C_{IS} = Concentration of internal standard (mg/ml).
V_{IS} = Volume of internal standard (□l).
□A_{Sterol} = Sum of the sterol peak areas.
A_{IS} = Peak area of the internal standard.
W_s = Weight of sample (mg)

For establishing the purity of the internal standard see paragraph 7.2.

11.3 Total Sterol Esters

C[Total Sterols] * (factor) = Total sterol esters

(factor) = ratio of MW of sterol ester/sterol * %conversion sterol to esters(typically 90%)
(factor) = 674/410 *0.9 = 1.479

12. RAW AND PROCESSED DATA

12.1 Raw data

The unprocessed (raw) data of an analysis contains:
- raw data files (on disk or tape),
- work sheets,
- notebook entries,
- hard copy sequence files.

12.2 Processed data

The processed data of an analysis contains:
- copy of analysis application form,
- copy of customer report,
- hard copies of "processed" data files.

13. PRECISION OF THE METHOD

13.1 Trueness

For the determination of the trueness MCT oil was spiked with 15 % (w/w) of a sterol ester concentrate.
The composition of the sterol ester concentrate was as follows:

Free sterols: 9.1 % (w/w)
Sterol ester: 90.9 % (w/w)
Total sterols: 9.1 % + (90.9 % x 0.608) = 64.4 % (w/w)

The results are given in table 3.

Table 3: Results trueness.

No.	Concentration of SE concentrate (%, w/w)	Theoretical S concentration (%, w/w)	Found S concentration (%, w/w)	Recovery (%)
1	15.17	9.77	9.78	100.1
2	15.08	9.71	9.66	99.5

In which:
SE = sterol ester
S = sterol

13.2 **Repeatability**

Samples of a margarine, halvarine, a fatblend and a sterol ester concentrate were analyzed 5 times (4 times for halvarine) at the same day.

13.2.2 Margarine

Table 4: Results repeatability margarine.

Repeat	**Total sterol content (%, w/w)**
1	10.27
2	10.30
3	10.30
4	10.25
5	10.30
Average	10.28
St. Deviation	0.02
Rel. St. Deviation	0.22

13.2.3 Halvarine

Table 5: Results repeatability halvarine.

Repeat	**Total sterol content (%, w/w)**
1	7.31
2	7.58
3	7.58
4	7.69
Average	7.54
St. Deviation	0.14
Rel. St. Deviation	1.86

13.2.4 Fat blend

Table 6: Results repeatability fat blend.

Sample	Total sterol content (%, w/w)
1	12.67
2	12.68
3	12.57
4	12.57
5	12.60
Average	12.62
St. Deviation	0.05
Rel. St. Deviation	0.42

13.2.5 Sterol ester concentrate

Table 7: Results repeatability sterol ester concentrate.

Sample	Total sterol content (%, w/w)
1	61.31
2	60.95
3	61.33
4	61.45
5	61.35
Average	61.28
St. Deviation	0.19
Rel. St. Deviation	0.30

13.2.6 Dressings

Table : Result Repeatability Dressings

Sample	Total sterol content (%, w/w)
1	3.77
2	3.81
3	3.56
4	3.80
5	3.76
Average	3.74
St. Deviation	0.10
Rel. St. Deviation	2.75

14. QUALITY ASSURANCE AND CONTROL

14.1 Blank sample

The first sample in an analysis batch is always a blank (MCT oil). No peaks should be detected in the GC chromatogram of the sterol analysis of this sample.

14.2 Check sample

In every analysis batch the check sample is analyzed. The results are logged in a control chart.

15. SPECIAL CASES AND NOTES

Table 8: Rounding off from the sterol results.

Total sterol content (%, w/w)	N decimals	Interval
0.10 – 8.00	2	0.01
8.0 – 80.0	1	0.1
80 – 100	0	1

16. REFERENCES

16.1 Sop 414; Determination of the sterol content of vegetable oils and fats with TLC-GC.

16.2 Sop 467; Determination of the sterol (4-desmethyl) content and composition in vegetable and animal oils and fats with off-line LC-GC.

17. ANNEXES

17.1 Annex 1: Example of the equipment settings.

17.2 Annex 2: Example of a GC chromatogram.

17.3 Annex 3: Table with relative retention times of some common sterols.

ANNEX 1

EXAMPLE OF THE SETTINGS OF THE EQUIPMENT

CHROMATOGRAPHIC SETTINGS STERTOT ANALYSIS SOP 462/01

Gas chromatograph		: GC 8000 series with AS-800 autosampler (akt.nr. 79629).
GC-column		: Chrompack, CP-Sil-5CB, 10 m * 0.32 mm, FD = 0.12 µm (Column number 74).
Precolumn		: Deactivated Fused Silica, ca. 0.5 m * 0.53 mm.
Column pressure (H_2)		: 60 KPa.
Column flow (H_2)		: 2,2 ml/min.
FID pressures	H_2	: 50 KPa.
	Air	: 100 KPa.

Oven programme

SB Time	: 0 min
Temp1	: 60 °C
Time1	: 1 min
Rate1	: 20 °C/min
Temp2	: 300 °C
Time2	: 3 min
Rate2	: 0 °C/min
Temp3	: 300 °C
Time3	: 0 min
Detector temp	: 320 °C (zone 1)
Sec. cool.	: 0 sec.
Oven limit	: 350 °C

ANNEX 1

EXAMPLE OF THE SETTINGS OF THE EQUIPMENT

AS-800 autosampler settings

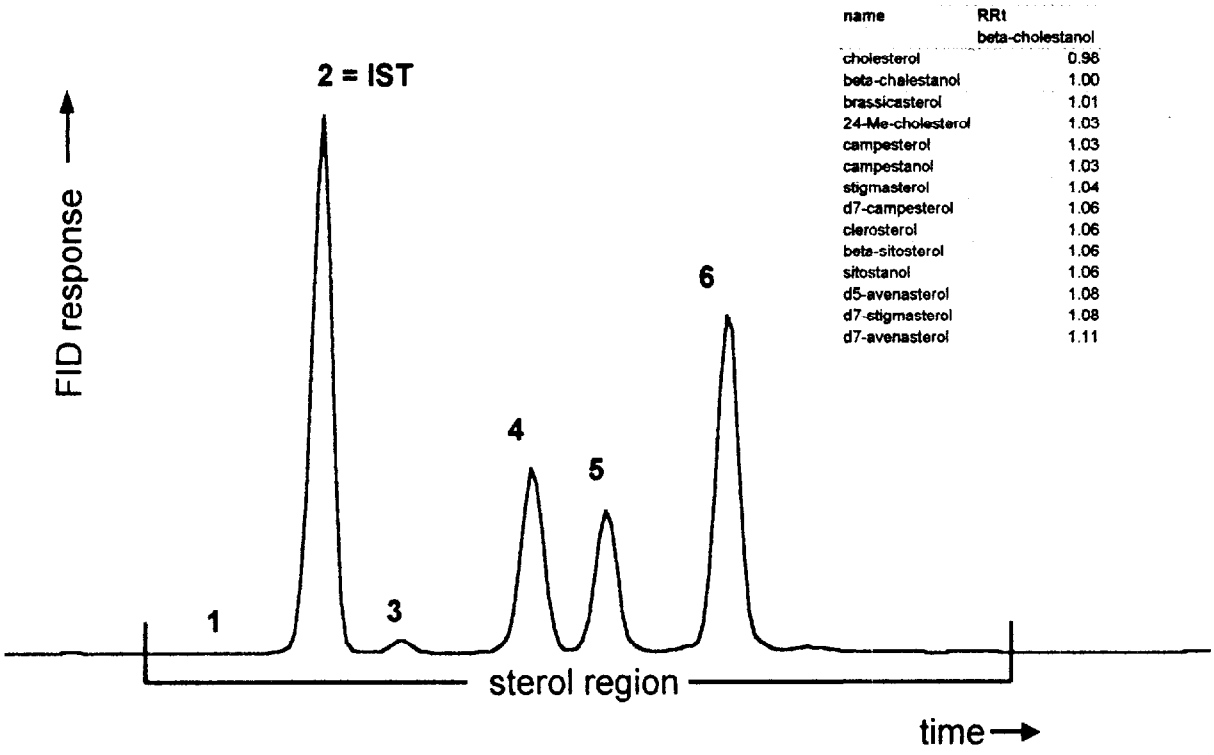
DESCRIPTION :		DISPLAY :				
Methode 0		METHOD A.0		Analysis T. OFF min		
Analysis time	: off	M.A0	Cnt	Mod	µl	S1
Pre-cleaning cycles	: 5	Pre.C	5	Inj	5.0	a
Cleaning mode	: inj					
Volume cleaning cycle	: 5 µl					
Solvent vial	: A					
Sample (injection volume)	: 1.0 µl	M.A0	Samp	Air	Fill	
Air volume	: 0.5 µl 3.0 µl	µl	0.5	1.0	5.0	Filling volume :
Cleaning cycles	: 5	M.A0	Cln	B.E.	µls	DI
B.E.	: 5	Fill.	5	5	5	1
Sample drawing speed	: 5 µl/sec					
Delay time	: 1 sec					
Speed injection	: max.	M.A0	µls	Pre	Post	
Pre-injection	: 1 sec	INJ.	Max	1s	5s	
Post-injection	: 5 sec					
Post-cleaning cycles	: 10	M.A0	Cnt	Mod	µl	s2
Cleaning mode	: inj	Post.C	10	Inj	5.0	a
Volume cleaning cycle	: 5 µl					
Solvent vial	: A					

Electrometer settings (EL-980)

Range	: 10°
Attenuation	: 2^4

ANNEX 2

EXAMPLE OF CHROMATOGRAM



name	RRt beta-cholestanol
cholesterol	0.98
beta-cholestanol	1.00
brassicasterol	1.01
24-Me-cholesterol	1.03
campesterol	1.03
campestanol	1.03
stigmasterol	1.04
d7-campesterol	1.06
clerosterol	1.06
beta-sitosterol	1.06
sitostanol	1.06
d5-avenasterol	1.08
d7-stigmasterol	1.08
d7-avenasterol	1.11

1 = Cholesterol
2 = □-Cholestanol (internal standard)
3 = Brassicasterol
4 = Campesterol + Campestanol
5 = Stigmasterol
6 = □-Sitosterol + Sitostanol

The unlabelled peaks are also sterols and must be included in the quantification.

ANNEX 3

RELATIVE RETENTION TIMES OF SOME COMMON STEROLS

Table 9 : Relative retention times of some sterols.

Sterol	**RRt** Relative to cholesterol	**RRt** relative to □-Cholestanol
Cholesterol	1.00	0.98
beta-cholestanol	1.02	1.00
Brassicasterol	1.03	1.01
24-Me-cholesterol	1.05	1.03
Campesterol	1.05	1.03
Campestanol	1.05	1.03
Stigmasterol	1.06	1.04
d7-campesterol	1.08	1.06
Clerosterol	1.08	1.06
beta-sitosterol	1.08	1.06
Sitostanol	1.08	1.06
d5-avenasterol	1.10	1.08
d7-stigmasterol	1.10	1.08
d7-avenasterol	1.13	1.11